INTRODUCTION

Two motor systems are known to generate cytoplasmic movements in eukaryotic cells. One is based on the interaction of myosin with microfilaments (MFs), and the other one is based on microtubules (MTs), which function as tracks for two different mechanochemical enzymes: kinesin and dynein (for review see Endow and Titus, 1992).

Actin filaments are distributed within the whole cytoplasm in the Angiosperm pollen tube, whereas myosin molecules are mainly located on the organelle surface. Actin filaments and myosin contribute to the organization of an energy transducing system that seems to have an active role in the transport processes during pollen germination and tube growth (for review see Pierson and Cresti, 1992).

MTs in the pollen tube have been investigated by different techniques, which revealed their distribution within the vegetative (VC) and generative cell (GC) cytoplasm (Pierson and Cresti, 1992; Del Casino et al., 1993). These observations led to several hypotheses on the role of MTs during pollen tube growth and in the GC. For instance, VC MTs could play a role in the intracellular movement of organelles during tube elongation (Pierson and Cresti, 1992). Furthermore, the GC MTs could be involved in the reshaping processes of the GC during its migration along the tube (Heslop-Harrison et al., 1988). MTs are also important in the division of the GC into the two sperm cells (for review see Palevitz and Tiezzi, 1992).

Microtubular motors in pollen tubes have been studied since an immunoreactive homolog of mammalian kinesin was discovered (Tiezzi et al., 1992) and biochemically characterized (Cai et al., 1993) in Nicotiana tabacum pollen tubes. Recent studies showed that a kinesin-related protein is associated with the vesicle surface in Corylus avellana pollen (Liu et al., 1994).

On the other hand, at present there is no evidence of dynein or dynein-like proteins in the pollen tube. Dynein has been found responsible for several intracellular movements associated with MTs (Endow and Titus, 1992). In cilia and flagella dynein is responsible for the generating force between the array of MTs organized in the axonemal structure. In addition, cytoplasmic dynein has been implicated in other forms of intracellular motility, including retrograde axonal transport, positioning of cytoplasmic organelles as lysosomes, endosomes and Golgi vesicles (for review see Schroer and Sheetz, 1991). Cytoplasmic dynein has been also implicated in the formation of the bipolar spindle (Vaisberg et al., 1993) and in some stages of the migration of chromosomes during mitosis (Pfarr et al., 1990).

Bidirectional movements of vesicles and organelles, and the GC migration and division processes, are observed during pollen tube growth. Therefore, to better understand the dynamic processes occurring within the pollen tube, the presence of MT-interacting proteins with ATPase activity related to that of dyneins was investigated.

We found that Nicotiana tabacum pollen tubes contain two high molecular weight polypeptides (about 400 kDa), which are specifically expressed during pollen germination and pollen tube growth in BK medium. The high molecular weight doublet resembles the dynein heavy chains in some biochemical properties. Sedimentation profiles of pollen tube extracts show that the high molecular weight bands have sedimentation coefficients of 22 S and 12 S, respectively. ATPase assay of sedimentation fractions shows an activity ten times higher when stimulated by the presence of bovine brain microtubules in fractions containing the 22 S high molecular weight polypeptide. Both these high molecular weight polypeptides can bind microtubules in an ATP-dependent fashion. A mouse antiserum to a synthetic peptide reproducing the sequence of the most conserved ATP-binding site among dynein heavy chains recognized the two high molecular weight polypeptides. Therefore these polypeptides have sequences immunologically related to the ATP binding sites of dynein heavy chains.

Key words: pollen tube, Nicotiana tabacum, synthetic peptide, dynein

SUMMARY

Nicotiana tabacum pollen tubes contain two high molecular weight polypeptides (about 400 kDa), which are specifically expressed during pollen germination and pollen tube growth in BK medium. The high molecular weight doublet resembles the dynein heavy chains in some biochemical properties. Sedimentation profiles of pollen tube extracts show that the high molecular weight bands have sedimentation coefficients of 22 S and 12 S, respectively. ATPase assay of sedimentation fractions shows an activity ten times higher when stimulated by the presence of bovine brain microtubules in fractions containing the 22 S high molecular weight polypeptide. Both these high molecular weight polypeptides can bind microtubules in an ATP-dependent fashion. A mouse antiserum to a synthetic peptide reproducing the sequence of the most conserved ATP-binding site among dynein heavy chains recognized the two high molecular weight polypeptides. Therefore these polypeptides have sequences immunologically related to the ATP binding sites of dynein heavy chains.

Key words: pollen tube, Nicotiana tabacum, synthetic peptide, dynein
high molecular weight polypeptides (about 400 kDa) whose expression is related to tube elongation. We show here that they exhibit some biochemical properties typical of dynein heavy chains such as electrophoretic migration, binding to brain MTs in an ATP-dependent fashion, sedimentation coefficient, and MT-stimulated ATPase activity. Furthermore, the two high molecular weight polypeptides in pollen tubes appeared immunologically related to dynein heavy chains, since they were recognized by a polyclonal antibody to a synthetic peptide reproducing the most conserved ATP binding site among dynein heavy chains.

**MATERIALS AND METHODS**

**Pollen germination**

*Nicotiana* pollen was collected from plants grown in the Botanical Garden of Siena University, dehydrated by incubation for 12 hours in a box containing silica gel and then stored at −20°C. Before germination, pollen grains were incubated for 15 minutes in ice, 15 minutes at 4°C, 15 minutes at room temperature and finally hydrated in a humid chamber for 1 hour. Pollen grains were then germinated in BK medium (Brewbaker and Kwack, 1963) containing 15% sucrose for periods of 45 minutes, 90 minutes, 3 hours and 15 hours at 25±1°C.

**Pollon fixation and tube length measurement**

Pollon tubes were fixed in 3% paraformaldehyde in PEM buffer containing 12% sucrose (for PEM composition, see below) for 30 minutes. Samples were rinsed three times for 5 minutes in PEM buffer and specimens were then prepared. Pollen tubes grown for different periods were observed with a MRC 500 (Bio-Rad) confocal laser scanning microscope and the tube length was measured using the ‘length option’.

**Polon and pollen tube extracts**

Materials were homogenized in two volumes of PEM buffer (100 mM PIPES, pH 6.8, 5 mM MgCl₂, 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml pepstatin A, 10 µg/ml TAME, 1 mM DTT) on ice, using a Potter homogenizer (25 strokes). After centrifugation at 4°C, 30 minutes, 15,000 rpm, in a Sorvall SS-34 rotor, the resulting supernatant was further spun at 4°C, for 1 hour at 45,000 rpm in a Sorvall T-865 rotor. The pellet was discarded whereas the high speed supernatant (HSS) was assayed for protein concentration by the Lowry procedure (Lowry et al., 1951), using BSA as the standard protein.

**Sedimentation of Nicotiana pollen tube high speed supernatant (HSS)**

Sucrose gradients (5% to 25%) were prepared in PEM buffer according to the method of Baxter-Gabard (1972). HSS (300 µl of 3 mg/ml of *Nicotiana tabacum* pollen tubes grown for three hours in BK medium) were loaded on each gradient. As a control, one gradient was used to sediment proteins with known sedimentation constants: thyroglobulin (19.1 S), catalase (11.3 S) and BSA (4.4 S). Gradients were centrifuged at 30,000 rpm in the Sorvall rotor AH-650 at 4°C for 15 hours and then each gradient was separated into 20 fractions. An equal volume of each fraction was denatured and analyzed by SDS-polyacrylamide gel electrophoresis.

**Purification of bovine brain cytoplasmic dynein**

Bovine brain (70 g) was homogenized in PEMEG buffer (100 mM PIPES, pH 6.8, 1 mM EGTA, 2 mM MgCl₂, 1 mM GTP, 10 µg/ml leupeptin, 1 mM PMSF) and then centrifuged at 23,000 rpm in a Sorvall T-865 rotor at 4°C for 30 minutes. The pellet was discarded and the supernatant centrifuged again at 37,000 rpm in the Sorvall T-865 rotor at 4°C for 1 hour. Hexokinase (10 U/ml) and glucose (50 mM) were added to the supernatant following an incubation for 20 minutes at room temperature to deplete the endogenous ATP content. To polymerize MTs, taxol (20 µM) and GTP (1 mM) were added and the sample was incubated for 30 minutes at room temperature. In these conditions cytoplasmic dynein bound to MTs, which were then collected by centrifugation (23,000 rpm in the Sorvall T-865 rotor, 15°C, 30 minutes). The pellet was resuspended in PEGE buffer containing 20 µM taxol and 1 mM GTP, and then centrifuged again in the same conditions. The pellet was then resuspended in PEGE containing 20 µM taxol and 10 mM ATP, then incubated for 30 minutes at room temperature. In this step cytoplasmic dynein was released from MTs and was collected in the supernatant after centrifugation at 37,000 rpm (Sorvall T-865 rotor, 15°C, 20 minutes). The cytoplasmic dynein fraction was loaded on 4.5 ml of a 5% to 25% sucrose gradient (Baxter-Gabard, 1972) and sedimented by centrifugation at 30,000 rpm (Sorvall AH-650 rotor, 4°C, 15 hours). Finally, 20 fractions of 10 drops each were obtained.

**Preparation of MTs from bovine brain**

MTs were assembled from purified brain tubulin by three cycles of temperature-dependent polymerization-depolymerization (Fellous et al., 1977). Microtubule-associated proteins (MAPs) were separated by anion exchange chromatography using a Mono Q column (Pharmacia) connected to a FPLC system, using an elution gradient of NaCl (0 to 1 M) in PEM containing 0.1 mM GTP. Tubulin was eluted at 0.5 M NaCl and then frozen in liquid nitrogen. Purity of the tubulin fractions was assayed by SDS-PAGE: no high molecular weight microtubule-associated proteins contaminated the tubulin preparation (data not shown). Before use, tubulin was incubated at room temperature for 30 minutes in the presence of 30 µM taxol. The sample was then centrifuged at 20,000 g for 30 minutes at 25°C. The supernatant was discarded and the pellet was resuspended in GTP-free PEM buffer.

**Binding assay of polypeptides from pollen tubes to bovine brain MTs**

The binding assay was performed according to Koonce and McIntosh (1990) with some minor modifications. *Nicotiana* pollen was germinated for three hours at room temperature in BK medium and then homogenized in an equal volume of PEGE buffer (100 mM PIPES, pH 7.0, 4 mM MgCl₂, 5 mM EGTA, 0.5 mM EDTA, 0.9 M glycerol, 10 µg/ml leupeptin, 5 µg/ml pepstatin A, 1 mM PMSF, 0.5 mM DTT), in ice. The homogenate was centrifuged at 15,000 rpm for 30 minutes at 4°C, in the Sorvall SS-34 rotor. The pellet was discarded and the supernatant centrifuged again in the Sorvall T-865, at 45,000 rpm for one hour at 4°C. The HSS was collected and used as follows. Hexokinase (10 U/ml) and D(+)-glucose (50 mM) were added to 1.2 ml of HSS (7 mg/ml protein concentration) and the mixture was incubated for 30 minutes at room temperature to deplete the endogenous ATP content. Bovine brain MTs were added to the HSS at a final concentration of 1 mg/ml. Taxol was also added at a final concentration of 10 µM. The mixture was incubated for 30 minutes at room temperature, following centrifugation at 18,000 rpm, for 30 minutes at 4°C in the Sorvall SS-34 rotor. The pellet was resuspended in 500 µl of PEGE buffer containing 10 µM taxol and centrifuged through 2 ml of 15% sucrose cushion in PEGE buffer containing 10 mM taxol (Sorvall AH-650 rotor at 20,000 rpm for 30 minutes at 4°C). The pellet was resuspended in 120 µl of PEGE buffer containing 10 µM taxol and 10 µM ATP, and incubated for 30 minutes at room temperature. The sample was then centrifuged in the SS-34 Sorvall rotor, 18,000 rpm, for 30 minutes at 4°C. The resulting supernatant was immediately denatured in Laemmli sample buffer. The pellet was rinsed again with PEGE buffer plus 10 µM taxol and resuspended in 120 µl of the same buffer containing 0.5 M NaCl (final concentration), and then incubated for 15 minutes at room temperature. The sample was finally centrifuged as reported above and both pellet and supernatant were denatured.
Production of the anti-peptide antibody

Sequences of the dynein heavy chains from several organisms were provided by the EMBL Data Bank (Heidelberg, Germany) and their comparison was performed using the BestFit program available at the Biocine Research Center (Siena, Italy).

From the comparative analysis one putative ATP binding site is apparently identical among all the observed sequences (shown in bold type hereafter); cytoplasmic dynein of *Dictyostelium discoideum* (MGGNPFGAPGKTETVKAFLG), cytoplasmic dynein from rat brain (LGGSFPGAPTGTKESEVKALG) and β-heavy chain of sea urchin dynein outer arm sperm flagella (MSGAPGAPGKTETKDLG). The sequence of the synthetic peptide used as antigen was MGGAPGAPGKTGT.

Solid-phase peptide synthesis was done in a Zinsser Analytic model (SMPS) 350 peptide synthesizer (Zinsser Analytic, Frankfurt, Germany) employing Fmoc chemistry. Coupling reactions were obtained with 1-hydroxybenzotriazole esters, monitoring for completeness by the acid-base indicator bromophenol blue. The peptide was cleaved from the resin and simultaneously deprotected using a trifluoroacetic acid/thioanisole/ethanedithiol/water (93:2:3:2, by vol.) mixture for two hours at room temperature. Purification was obtained by RP-HPLC with a Vydac C18 column. The amino acid composition analysis was performed on a Hitachi L-8800 amino acid analyser. The peptide was cleaved from the resin and simultaneously deprotected using a trifluoroacetic acid/thioanisole/ethanedithiol/water (93:2:3:2, by vol.) mixture for two hours at room temperature.

Three days after the third injection, ascitic fluid was collected from each mouse and two weeks the same treatment was repeated for each mouse.

**Preparation of Chlamydomonas reinhardii axonemes**

Cell cultures of wild type and the *pf* 28 mutant strain of *Chlamydomonas reinhardii* (Mitchell and Rosebaum, 1985) were grown on solid medium (Luck et al., 1977). Axonemes were prepared from wild type and from the *pf* 28 mutant by the dibucaine method (Witman, 1986).

**Electrophoresis and western blotting**

All the samples were denatured with Laemmli sample buffer and separated on 3% to 7% polyacrylamide gels using the method of Laemmli (1970). All gels were silver stained (Dunn, 1989). Molecular weight standards were purchased from Bio-Rad.

Western blots were performed according to Towbin et al. (1979). Polypeptides were also transferred to nitrocellulose using 10 mM CAPS (3-cyclohexylamino-1-propanesulfonic acid), 10% methanol, pH 11, at 250 mA for three hours. We used the following battery of monoclonal antibodies against dynein heavy chains of sea urchin flagella: C-241-2, 132-7 and C-26-2 (Piperno, 1984), kindly provided by Prof. G. Piperno (Mount Sinai School of Medicine, NY) and the mouse polyclonal antibody to the synthetic peptide (see below). As secondary antibody, a goat anti-mouse peroxidase conjugate (1: 4000, final dilution) was purchased from Cappel.

**ATPase activity assay**

ATPase assays in the absence and presence of bovine brain MTs were done in PEM buffer, according to the colorimetric procedure of Gonzalez-Romo et al. (1992) with some minor modifications as already reported by Cai et al. (1993).

**RESULTS**

**Presence of high molecular weight polypeptides in Nicotiana pollen tubes**

Dyneins are complex proteins comprising high molecular mass polypeptides (heavy chains) of about 400 kDa and several additional polypeptides called intermediate and light chains (Witman, 1989). Portions of the protein interacting with the microtubules and carrying the ATP hydrolysing sites in all dyneins are located in the heavy chain polypeptides.

Since the presence of heavy chains (400 kDa) represents one characteristic aspect of dynein molecules, the HSS from *Nicotiana tabacum* pollen tubes grown for three hours in BK medium was analysed by SDS-PAGE to see if any polypeptides with a molecular weight in the range of 400 kDa were present (Fig. 1, lane d).

HSS from *Nicotiana* pollen tubes grown for three hours in BK medium contain some polypeptides with a molecular weight higher than 200 kDa (Fig.1, lane d). To obtain more information on the size of the pollen tube’s high molecular weight polypeptides, a polyacrylamide gel electrophoresis of bovine brain cytoplasmic dynein (a), wild-type and *pf* 28 mutant *Chlamydomonas* axonemes (b,c), and *Nicotiana tabacum* pollen tube extract (d) was done in PEM buffer.

**Fig. 1.** SDS-polyacrylamide gel electrophoresis of bovine brain cytoplasmic dynein (a), wild-type and *pf* 28 mutant *Chlamydomonas* axonemes (b,c), and *Nicotiana tabacum* pollen tube extract (d). A polypeptic doublet of about 400 kDa is present in the tobacco pollen tube extract. Profiles of Mr standards are shown in lane S.
weight polypeptides, they were compared with the dynein heavy chains from *Chlamydomonas* axonomes and from calf brain. The position in the gel of *Chlamydomonas* outer arm dynein heavy chains has been identified by comparing the electrophoretic profile of wild-type axonomes (Fig. 1, lane b) with that of the pf 28 mutant, which lacks outer arm dynein heavy chains (Fig. 1, lane c). The comparison with the electrophoretic pattern of flagellar polypeptides from the two *Chlamydomonas* strains showed that pollen tube extracts contained two high molecular weight polypeptides (named HMW doublet; Fig. 1, lane d, see arrows) migrating near the outer arms dynein heavy chains of *Chlamydomonas* axonomes (400 kDa; absent in pf 28 mutant) (Mitchell and Rosebaum, 1985). The calf brain cytoplasmic dynein heavy chain showed higher electrophoretic mobility when compared with that of the HMW doublet from *Nicotiana tabacum* pollen tubes (Fig. 1, lane a). In all the pollen tube extracts analysed by electrophoresis, the faster migrating band of the HMW doublet appeared more intense than the slower migrating polypeptide.

**Binding of the pollen tube HMW doublet to bovine brain MTs**

Dyneins are microtubule interacting proteins and it has been shown in several organisms (Koonce and McIntosh, 1990; Moss et al., 1992; Porter and Johnson, 1983) that they are able to bind microtubules in the absence of ATP and are released after incubation with ATP.

The ability of the pollen tube HMW bands to bind reversibly to bovine brain MTs was tested and the results are reported in Fig. 2. In the HSS the high molecular weight doublet was still visible in the gel after incubation with hexokinase (Fig. 2, lane c, indicated by arrowheads). During this step, the lower molecular weight component of the HMW doublet appeared to be partially degraded when compared with the amount present before incubation with hexokinase (lane not shown). Taxol-stabilized MTs were prepared from purified calf brain tubulin and added to the pollen tube HSS in the absence of ATP. Under these conditions both HMW bands appeared to bind to the exogenous MTs, together with other lower molecular weight polypeptides (Fig. 2, lane b). The HMW doublet was significantly enhanced in the MT-pellet with respect to other polypeptides, when compared with the starting HSS (lane a). The same experiment performed without an exogenous microtubule pellet did not induce sedimentation of the doublet (data not shown).

To test if the interaction of the HMW doublet with MTs was ATP sensitive, the MTs pellet of lane b was resuspended in PMEG buffer containing 10 mM ATP. Fig. 2 shows polypeptides extracted in the above described conditions and those that remain bound to MTs after ATP incubation (lanes c and d, respectively). The ATP induced a consistent but incomplete extraction of the HMW bands (lane c, arrows), since parts of them remain bound to MTs (lane d). The pellet of lane d was further resuspended in PMEG buffer containing 0.5 M NaCl, to induce the dissociation of polypeptides that were eventually bound to bovine brain MTs by ionic interactions. The HMW doublet is visible in the salt-extracted fraction (Fig. 2, lane e). No high molecular weight polypeptide is still associated with the final MT pellet (Fig. 2, lane f). Therefore, these data indicate that the pollen tube HMW polypeptides are part of proteins which have both sensitive and insensitive binding sites for microtubules.

**Sedimentation and ATPase activity**

One of the most used criteria for the identification of dynein polypeptides has been the cosedimentation of polypeptidic components with ATPase activity because dyneins are ATPases which sediment between 10 and 30 S. Sedimentation coefficients of the HMW doublet were calculated by zonal centrifugation experiments through sucrose gradients, comparing their positions with those of standard proteins. The sedimentation profile of proteins present in the gradient fractions was analyzed by SDS-PAGE and is shown in Fig. 3A. The two high molecular weight polypeptides of the doublet were present in two sets of fractions. Fractions 5-7 contained the higher molecular weight band of the doublet (see arrowhead in A) whose sedimentation coefficient appeared to be in the range of 22 S (referred to as the ‘22 S band’). The lower molecular weight polypeptide of the doublet was found in fractions 12-14 (see arrowhead), showing a sedimentation coefficient of 12 S (the ‘12 S band’). The sedimentation coefficient of both high molecular weight polypeptides was estimated on the basis of three different sedimentation experiments, comparing their position in the gradient to that of three standard proteins: thyroglobulin (19.1 S), catalase (11.3 S) and BSA (4.4 S).

The sedimentation fractions were also assayed for their ATPase activity in the absence and presence of bovine brain MTs. The enzymatic activity data are shown in Fig. 3B. Fractions 5-7, containing the 22 S band, did not show any significant ATPase activity in the absence of MTs (1.5 nmoles P_i/min per mg, in fraction 7), whereas activity (to 15.3 nmoles P_i/min per mg, in fraction 7, asterisk) was observed in the
presence of bovine brain MTs. Fractions containing the 12 S band (fractions 12-14) revealed no significant stimulation of ATPase activity in the presence of added MTs.

**Reactivity of the anti-synthetic peptide antibody**

An immunological relation between the HMW doublet of *Nicotiana tabacum* pollen tube and dynein heavy chains could not be shown using several monoclonal antibodies against the dynein heavy chains of sea urchin sperm flagella (Piperno, 1984; and data not shown). In order to identify epitopes which were common among dynein heavy chains from several organisms a comparison between the dynein heavy chain sequences that have been published was made. On the basis of this sequence analysis, a polyclonal antibody was elicited against the synthetic peptide reproducing the most conserved putative ATP binding site found in all dynein heavy chains from different organisms (see Materials and Methods). The reactivity of the anti-synthetic peptide polyclonal antibody was initially tested by a spot-test on the same peptide used as antigen. In that case, the synthetic peptide was conjugated with BSA instead of KLH. The use of conjugated peptide-KLH would make it difficult to evaluate the positive reaction of the antiserum to the peptide, since it also probably contains a small amount of antibodies to KLH. BSA activated with glutaraldehyde and bound to the synthetic peptide (Fig. 4, row A) and BSA activated with glutaraldehyde (Fig. 4, row B) were absorbed on nitrocellulose. The polyclonal antibody strongly recognized the sample in which the synthetic peptide was present (Fig. 4, row A) and only a feeble positive reaction, not visible in the picture, was observed in Fig. 4, row B. Control experiments were carried out on the same samples, employing, in one case, an anti-tubulin antibody (Fig. 4, row C and D) and, in the second case, only the secondary antibody (Fig. 4, rows E and F). Both control assays gave negative reactions.

The antibody was further tested on calf brain cytoplasmic dynein. In Fig. 5, lanes a-d show different stages of the calf
brain cytoplasmic dynein purification, whereas lane e shows the positive reaction of the anti-synthetic peptide polyclonal antibody to the calf brain cytoplasmic dynein heavy chain (lane d, arrowhead). The antibody did not recognize the intermediate and light chains of the protein which were visible in the gel.

The reactivity of the antibody with the pollen tube HMW doublet was assayed on the MT pellet obtained after the binding of the pollen tube polypeptides to MTs (Fig. 6, lane a). The antibody specifically recognized the two high molecular weight bands which were able to bind to MTs (Fig. 6, lane b, arrows).

**Presence of the HMW doublet in pollen and pollen tubes during germination**

In order to determine if the presence of the high molecular weight doublet on pollen is related to pollen germination and pollen tube growth, the presence of the high molecular weight polypeptides was studied during pollen hydration and pollen tube elongation in BK medium. The corresponding electrophoretic profiles of pollen and pollen tube extracts are shown in Fig. 7 (90 µg of protein was loaded in each lane). In dry pollen, polypeptides above 200 kDa are absent (lane b), whereas after one hour of hydration, new polypeptides with molecular weights higher than 200 kDa are present (lane c). In particular, the HMW doublet can be seen (lane c, see arrowheads). After three hours of germination in BK medium, the intensity of the same doublet increased, and the enhancement was more prominent for the band with lower molecular weight (lane d). After 15 hours of germination, only the lower molecular weight band of the doublet was still visible in the gel (lane e).

In order to understand if the presence of the HMW doublet in pollen tubes is related to the pollen tube length, the presence of the doublet was investigated in pollen tubes grown in BK medium for different times. Pollen grains were germinated in BK medium and samples were taken at different periods. The lengths of pollen tubes grown in BK medium were measured and the corresponding electrophoretic patterns were observed after 45 minutes, 90 minutes and three hours. Pollen tube lengths and electrophoretic profiles are both reported in Fig. 8. The electrophoretic profiles of pollen tubes germinated in BK medium for 45 minutes, 90 minutes and three hours are reported in Fig. 8 (lanes b, c and d, respectively). The HMW
doublet (arrows in b) was visible in the gel at the three different times even if with different intensities. After 45 minutes of germination (when most of the pollen tubes are 60-100 µm long), the doublet appeared to be more pronounced than after 90 minutes and three hours.

DISCUSSION

This paper reports that *Nicotiana tabacum* pollen tubes contain a HMW doublet that shares several properties with dynein heavy chains: (a) the HMW doublet coelectrophoreses with outer arm dynein heavy chains of *Chlamydomonas* axonemes and with bovine brain cytoplasmic dynein heavy chain on SDS-polyacrylamide gels; (b) it binds and copellets with microtubules in an ATP-sensitive fashion; (c) the two HMW polypeptides of the doublet are part of the proteins which sediment as 22 S and 12 S particles on sucrose density gradients; (d) an ATPase activity was observed in the 12 S fractions whereas an ATPase activity in the 22 S fractions was revealed only after addition of exogenous microtubules; (e) both components of the HMW doublet of *Nicotiana tabacum* pollen tube have epitopes immunologically related to the ATP binding sites of dynein heavy chains.

Furthermore, our observations clearly show that the presence of the HMW doublet in pollen tubes can be related to pollen germination and tube growth.

Together these data suggest that *Nicotiana tabacum* pollen tubes contain MT motor proteins related to dyneins.

**Biochemical properties of the HMW doublet**

The presence in pollen tube extracts of polypeptides with a molecular weight in the range of 400 kDa (Fig. 1) made it an attractive prospect to investigate the presence of proteins similar to dyneins in higher plants. The pollen tube HMW doublet showed biochemical characteristics typical of axonemal and cytoplasmic dynein heavy chains. The ability of dyneins to interact with MTs in an ATP-dependent fashion has been reported (Koonce and McIntosh, 1990). An ‘in vitro’ binding experiment using calf brain MTs revealed that the pollen tube HMW doublet binds to exogenous MTs and is partially released in the presence of ATP. Part of the doublet still remains bound to MTs and is detached only when MTs are incubated with NaCl. These data suggest that both the 12 S and 22 S bands in *Nicotiana* pollen tubes could be related to proteins interacting with MTs at both an ATP-sensitive and an insensitive site, as reported for sea urchin egg dynein (Hisanaga and Sakai, 1983).

Sedimentation of a high speed supernatant from pollen tubes through a sucrose gradient showed that the higher and lower molecular weight bands of the doublet have sedimentation coefficients of 22 S and 12 S, respectively. The same sedimentation constants are common to the ciliary outer arm dyneins identified in *Paramecium tetraurelia* (Beckwith and Asai, 1993) and to the C/A dynein recently identified in the flagellar axonemes of sea urchin sperm (Yokota and Mabuchi, 1993).
A sedimentation coefficient of 21-22 S has also been determined for the ciliary outer arm dynein of *Tetrahymena* (Mitchell and Warner, 1981). A 12 S value has been found for the *Chlamydomonas* (Fay and Witman, 1977), porcine tracheal ciliary (Hastie et al., 1986) and bull spermatozoa outer arm dyneins (Belle Isles et al., 1986). There is a small difference between the sedimentation coefficient values of HMW bands from pollen tubes and those of the identified cytoplasmic dyneins which are in the range of 20 S, like those in sea urchin egg (Grissom et al., 1992), *Dictyostelium* (Koonce and McIntosh, 1990), *Caenorhabditis elegans* (Lye et al., 1987) and the mammalian nervous system (Paschal et al., 1987).

The ATPase assays demonstrated a level of enzymatic activity corresponding to the 12 S band fractions, which did not seem to be stimulated by the presence of added MTs. On the other hand, in the 22 S band fractions, an ATPase activity, normally latent, proved to be 10 times activated in the presence of exogenous MTs. The low level of activity in the 22 S fractions in the absence of MTs may be due to the lack of a specific ‘substrate’ for the enzyme. Dyneins are known to be MT-activated ATPases and an enhancement of their enzymatic activity in the presence of MTs has been shown already; calf brain cytoplasmic dynein, which normally has a low ATPase activity, is stimulated 4-fold by the presence of MTs (Paschal et al., 1987). The C/A dynein recently isolated from sea urchin sperm flagellar axonemes is 6- to 7-fold stimulated by the presence of MTs (Yokota and Mabuchi, 1994a,b). The difference in stimulation between the 22 S and 12 S fractions in the presence of bovine brain MTs may suggest structural or functional differences between the two HMW bands. The absence of activation by MTs in the 12 S fractions could also be explained by supposing that other ATPases cosediment in the 12 S fractions and partially mask an eventual stimulation by MTs. Alternatively, there is the possibility that small tubulin polymers present in the pollen tube extract can cosediment within the 12 S fractions and, in that case, the ATPase activity revealed in the absence of bovine brain microtubules could represent ATPase activity already stimulated by MTs.

**Anti-synthetic peptide polyclonal antibody**

Complete amino acid sequences of dynein heavy chains from different organisms have been published in recent years: sea urchin sperm flagellar axonemes (Gibbons et al., 1991; Ogawa, 1991), *Dictyostelium* cytoplasmic dynein (Koonce et al., 1992) and rat brain cytoplasmic dynein (Zhang et al., 1993). Sequence comparison shows that the polypeptide homology is not high. The sequence identity between cytoplasmic and axonemal dyneins is around 27%, whereas the degree of conservation for the same functional form of dynein tends to be higher (54% between rat and *Dictyostelium* cytoplasmic dynein; Vallee, 1993). The most remarkable common features of the several dynein heavy chains are four P-loop consensus sequence elements. The region surrounding the first P-loop represents the most conserved part of the molecule and, on the basis of some biochemical properties, it has been suggested that it is involved in the ATP hydrolysis step (Hisanaga and Sakai, 1983). The sequence of the synthetic peptide was chosen by comparing the portions flanking the P-loop consensus sequence (GPAGTGKT) of dynein heavy chains with those of other ATPases like kinesin and myosin. In order to avoid eventual cross-reactions with those proteins, the part that lies ahead of the P-loop consensus element was chosen and the amino acids more frequently found among the dynein sequences were taken into consideration. The polyclonal antibody obtained specifically recognized the synthetic peptide used as antigen (Fig. 4) as well as the calf brain cytoplasmic dynein heavy chain (Fig. 5) and both the HMW polypeptides (22 S and 12 S) of the pollen tube (Fig. 6). The cross-reactivity data clearly show that both HMW bands of *Nicotiana* pollen tubes possess sequences that are immunologically related to the putative ATP binding site of dynein heavy chains from other organisms.

**The presence of the HMW doublet during germination**

The presence of these HMW polypeptides was investigated during germination. They were not present in dry pollen but their presence was detected when pollen grains were activated during hydration. After three hours of germination, when pollen tubes were actively growing, the intensity of the HMW doublet increased, with the lower molecular weight band of the doublet prominently enhanced. The amount of the higher molecular weight band remains apparently constant during hydration and three-hour germination whereas after 15 hours of germination only the lower molecular weight band is still visible in the gel (Fig. 7).

In order to better understand if the expression of the high molecular weight bands in pollen tubes can be related to the tube length, pollen grains were germinated in BK medium for different periods. Our observations clearly show that the presence of the high molecular weight polypeptides was more pronounced after 45 minutes of germination, when most of the tubes had a length of 60-100 µm long. After 90 minutes (100-140 µm) and three hours (260-300 µm) of germination, the intensity of the doublet appeared to be nearly the same.

These data suggest that the presence of the proteins carrying the two HMW polypeptides could be related to pollen tube growth and they appear to be very important in processes occurring during the first stages of pollen tube elongation.

**Conclusions**

The presence of dynein-related proteins in pollen tube opens new perspectives for understanding the role of MTs during pollen tube growth. The recently discovered kinesin immunoreactive homolog in pollen tube (Tiezzi et al., 1992) suggests that MTs can play a role in the complex cytoplasmic movement during pollen tube elongation. It is known that the pollen tube cytoplasm is highly organized and that a precise organelle distribution is maintained along the tube (Pierson and Cresti, 1992). Dynein-related proteins in pollen tubes could be involved in positioning organelles in the correct spatial arrangement within the tube and in the recycling of membranes among the different membranous networks during pollen tube elongation, in order to maintain the polar organization of the pollen tube cytoplasm.

On the other hand, they could also be important in microtubule sliding processes inside the generative cell (GC), which should allow GC reshaping (Heslop-Harrison et al., 1988) during its migration along the tube. Furthermore, MT-based motors could play a central role in the division of the GC in the two sperm cells.

Experiments are in progress to investigate the cytological localization of the proteins containing the two HMW polypep-
tides in order to elucidate their function as microtubule-interacting proteins with ATPase activity in pollen tubes.

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